ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Production and Partial Purification of Cellulase from Bacteria Inhabiting Cow Dung

Jahir Alam Khan[1*] Dilip Kumar[2]

[1]R & D Division, MRD LifeSciences (P) Ltd., Lucknow, India.
[2]Sai Nath Group of Education, Agra, India.

ABSTRACT

Three bacterial isolates namely MJD1101, MJD1102 and MJD1103 were isolated from Cow dung sample. All the three were subjected to cellulase screening and the isolate MJD1101 was formed to be positive. Isolate was able to grow at 37 °C and pH 7. Cellulase enzyme was produced by submerged fermentation and partially purified by salt precipitation and dialysis. Pure enzyme showed a good cellulase activity of 0.008 U/ml/min.
Keywords: Cellulase, Submerged fermentation, Salt precipitation, Dialysis.

Corresponding author
Email: jahir_84@rediffmail.com

ISSN: 0975-8585

INTRODUCTION

Cellulases are enzymes capable of degrading cellulose, a carbohydrate that is the essential and characteristic structural substance of the plant world. Some parts of plant system are of industrial importance but a major part is the food material of herbivore and other animal species. If converted to lower sugar forms these cellulose can be of major importance to the biofuel and other industries.

Cellulases have been reported [1] to be of different types; endoglucanase (1, 4-â-d-glucan-4-glucanohydrolase; EC 3.2.1.4), exocellobiohydrolase (1,4-â-d-glucan glucohydrolase; EC 3.2.1.74) and alpha-glucosidase (alpha-d-glucoside glucohydrolase; EC 3.2.1.21). Cellobiose /beta-galactosidase: It hydrolyses the exo-cellulase product into individual monosaccharide. Oxidative cellulase: It depolymerise cellulose by radial reactions, as for instance cellobiose dehydrogenase (acceptor). Cellulose phosphorylases: It depolymerise cellulose using phosphate instead of water.

Cellulases have attracted much interest because of the wide range of their application. The major industrial applications of cellulases are in textile industry for 'bio-polishing' of fabrics and producing stonewashed look of denims, as well as in household laundry detergents for improving fabric softness and brightness [2]. They are also used in animal feeds for improving the nutritional quality and digestibility, in processing of fruit juices, and in baking, while de-inking of paper is yet another emerging application. A potential challenging area where cellulases would have a central role is the bioconversion of renewable cellulosic biomass to commodity chemicals [3].

Cellulase can be produced by fungi, bacteria or actinomycetes but bacteria which have a high growth rate as compared to fungi have good potential to be used in cellulase production [4]. Cellulases have been reported to be produced from bacteria by various researchers [1; 4-7]. Looking at previous research on cellulase production by bacteria the present investigation was designed to evaluate cow dung as source of cellulase producing bacteria with following objectives (a) To screen the bacterial isolates from cow dung for cellulase production, (b) To optimize the growth parameters (growth curve, pH, temperature) of the isolate (c) Production and partial purification of cellulase (d) Characterization of purified cellulase.

MATERIALS AND METHODS

Isolation of Bacteria from Cow Dung

Bacterial isolates were isolated from Cow Dung by serial dilution agar plating method wherein Cow Dung sample was serially diluted upto 10^{-5} dilution and spread on sterile NA plates. Plates were incubated at 37 °C. Mixed cultures obtained were differentiated based on colony morphology and named tentatively as MJD1101, MJD1102 and MJD1103. All the three cultures were sub-cultured by quadrant streaking.

Screening for Cellulase Production

All the three isolates were subjected to cellulase screening by streaking them on MAM (minimal agar media) plates supplemented with 1% CMC. After incubation plates were flooded with 0.1 % Congo red dye and destained with 1M NaCl. Plates were then observed for zone of cellulose hydrolysis.

Staining and Biochemical Characterization of Isolate MJD1101

The isolate MJD1101 showing maximum cellulose hydrolysis was characterized by the help of staining (Gram's staining and Endospore staining) and Biochemical activities (Catalase test, Mannitol fermentation test) [8].

Study of Growth Parameters of Culture Positive in Screening

The isolate MJD1101 showing cellulose hydrolysis was selected for further studies and its growth parameters were studied.

1. **Growth Curve**

   In order to have an idea of the duration at which stationary phase (known for maximum secondary metabolite production) is reached by the isolate MJD1101, it was inoculated in of sterile NB (nutrient broth). And the growth of isolate was tracked by reading the absorbance of the inoculated media at 600 nm against uninoculated blank.

2. **Effect of Temperature**

   In order to have an idea of optimum temperature for the growth of isolate MJD1101, it was streaked on NA plates and incubated at different temperatures i.e. 22 °C, 28 °C 37 °C, 50 °C. Growth of isolate was judged.

3. **Effect of pH**

   In order to have an idea of optimum pH for the growth of isolate MJD1101, it was inoculated in NB of varying pH i. e 5, 7, 9, 11 and incubated at 37 °C for 24 hours at 120 rpm. Growth of isolate was tracked by reading the absorbance of the inoculated media at 600 nm against uninoculated blank.

Production of Cellulase by Submerged Fermentation

Cellulase was produced by inoculating 100 ml production media () with 1 ml of 24 hour old grown broth of isolate MJD1101. Inoculated flask was incubated at 37 °C for 7 days.

Extraction of Crude Enzyme

Crude cellulase was extracted from fermented broth by filtering the broth with whatman's filter paper and centrifuging the filtrate at 5000 rpm for 5 minutes at 4 °C. Supernatant was collected and treated as crude cellulase enzyme.

Partial purification of Crude Enzyme

Crude cellulase enzyme was partially purified by the help of ammonium sulphate precipitation upto 70 % saturation and dialysis in order to remove the residual salts.

Estimation of Protein in Crude and Partially Purified Enzyme

Concentration of protein in crude and purified enzyme was determined by Lowry's method [9] of protein estimation wherein 0.1 ml of enzyme was reacted with Lowry's reagents and reading the absorbance at 660 nm. Absorbance reading was compared to the standard graph prepared by reacting standard protein BSA **(0.02 – 0.2 mg/ml)** with Lowry's reagents.

DNS assay of Crude and Partially Purified Enzyme

CMCase activity was assayed using a standard method [10]. Different concentrations of glucose in a range of 0.05 to 0.5 mg/ml were reacted with Dinitrosalicylic acid and absorbance readings at 540 nm were recorded. A standard graph was plotted between concentration of glucose in X axis and absorbance at 540 nm in Y axis. The activity was estimated using 1% solution of carboxymethyl cellulose (CMC) in 0.1M Tris buffer (pH 7.0) as substrate. The reaction mixture contained 0.5 ml substrate and 0.5 ml of enzyme. The reaction was carried out at 37^{o}C for 15 min. The amount of reducing sugar released in the hydrolysis was measured. One unit of CMCase activity was defined as amount of enzyme required to release 1 micromoles of glucose per minute under standard reaction conditions.

Characterization of Partially Purified Enzyme

Purified enzyme was characterized for the effect of temperature and pH wherein enzyme assay was carried out at different temperature and pH using same DNS method described earlier.

RESULTS

Isolation of Bacteria from Cow Dung

Three bacterial isolates differing in colony morphology tentatively named as MJD1101, MJD1102, and MJD1103 were sub cultured by quadrant streaking and subjected to cellulase screening.

ISSN: 0975-8585

Screening for Cellulase Production

All the three isolates were subjected to cellulase screening and out of the three isolates, isolate MJD1101 was found to be positive after Congo red staining and distaining with 1M NaCl. Table 1 and Figure 1 below shows results of screening.

Table 1: Cellulase Screening

S. No.	ISOLATE	REMARKS
1.	MJD1101	+++
2.	MJD1102	-
3.	MJD1103	-

Figure 1: Cellulase Screening

Staining and Biochemical Characterization of Isolate MJD1101

The isolate MJD1101 was found to be Gram +ve cocci, positive in endospore staining, Catalase test and mannitol fermentation test.

Study of Growth Parameters of Culture Positive in Screening

Growth parameters (growth curve, temperature and pH) were studied in order to have an idea of the time when stationary phase is reached, the optimum temperature and pH for growth of the isolate MJD1101.

a) Growth Curve

Growth curve was studied in order to have an idea of the time when stationary phase is reached as stationary phase has been known to be best for production of secondary metabolites. Table 2 and Figure 2 below shows the absorbance readings and growth curve respectively.

ISSN: 0975-8585

Table 2: Growth Curve

S. No.	TIME (IN DAYS)	OD AT 600 nm
1	0	0
2	1	0.54
3	2	0.62
4	3	0.69
5	4	0.75
6	5	0.84
7	6	0.75
8	7	0.75
9	8	0.65
10	9	0.45

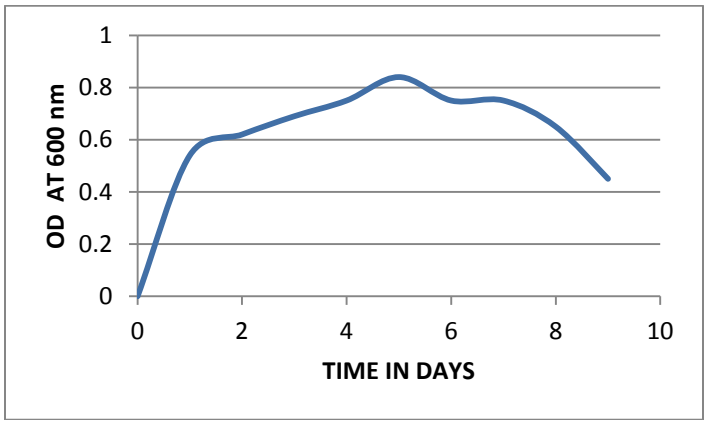

Figure 2: Growth Curve

b) Effect of Temperature

Effect of temperature on growth of isolate MJD1101 was studied in order to have an idea of the optimum temperature so that the same temperature can be used during production of cellulase. Table 3 below shows data of temperature optimization studies.

Table 3: Effect of Temperature

S. No.	INCUBATION TEMPERTURE	REMARKS
1	22 °C	-
2	28 °C	+++
3	37 °C	+++
4	50 °C	-

c) Effect of pH

Effect of pH on growth of isolate MJD1101 was studied in order to have an idea of the optimum pH so that the same pH can be used during production of cellulase. Table 4 and Figure 2 below show the results of the same.

ISSN: 0975-8585

Table 4: Effect of pH

S. No.	pH OF MEDIA	OD AT 600 nm
1	5	0.01
2	7	1.09
3	9	0.76
4	11	0.0

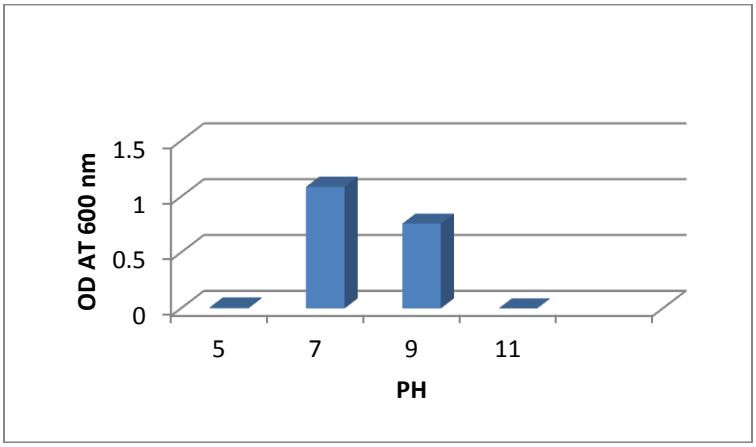

Figure 2: Effect of pH

Estimation of Protein in Crude and Partially Purified Enzyme

Concentration of protein in crude and partially purified enzyme was estimated by Lowry's method of protein estimation. Table 5 and Figure 3 below show the results of the same.

Table 5: Protein Estimation in Crude and Partially Purified Enzyme

TEST TUBES	ENZYME (IN ml)	DISTILLED WATER (IN ml)	REAGENT C (IN ml)	INCUBATED AT ROOM TEMP. FOR 15 MINUTES	REAGENT D (IN ml)	INCUBATED FOR 30 MINUTES IN DARK	O.D	CONC. OF PROTEIN (mg/ml)
BLANK	0.0	1	5		0.5		0.0	0.0
Crude Enzyme	0.5	0.5	5		0.5		0.27	0.098
Pure Enzyme	0.5	0.5	5		0.5		0.20	0.073

ISSN: 0975-8585

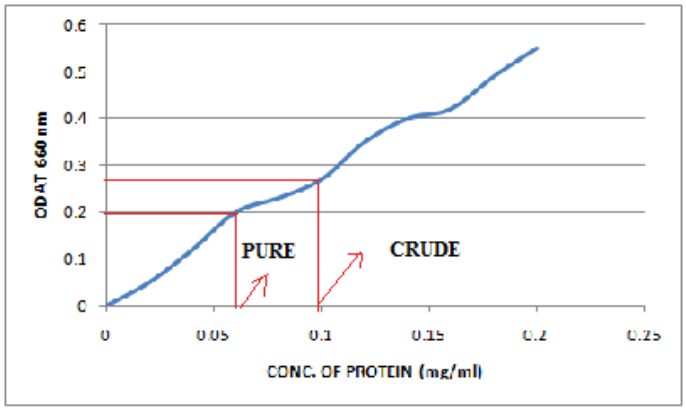

Figure 3: Protein Estimation in Crude and partially Purified Enzyme

DNS assay of Crude and Partially Purified Enzyme

Enzyme activity in crude and partially purified enzyme was done by DNS method and Table 6 and Figure 4 below show the result of DNS assay.

Table 6: Enzyme Assay of Crude and Partially Purified Enzymes

TEST TUBES	ENZYME (in ml)	1% STARCH (in ml)	INCUBATED AT 28°C FOR 15 MINUTES	DNS (in ml)	BOIL FOR 15 MINUTES AT 100°C	O.D AT 540 nm	Enzyme activity (U/ml/min)
BLANK	0	0		1		0.0	0.0
Crude Enzyme	0.5	0.5		1		0.28	0.010
Pure Enzyme	0.5	0.5		1		0.23	0.008

ISSN: 0975-8585

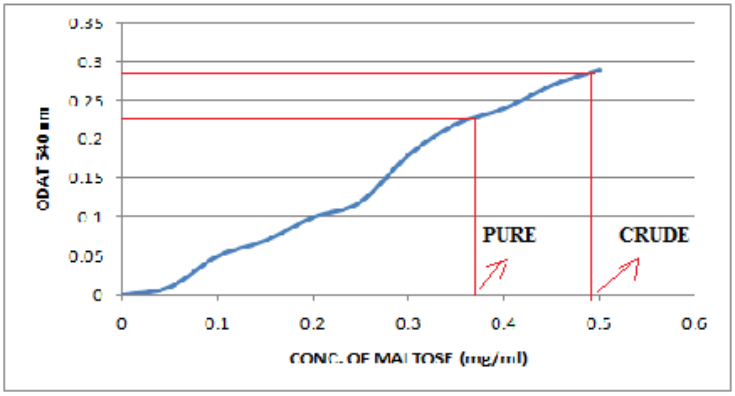

Figure 4: Enzyme Assay of Crude and Partially Purified Enzymes

CHARACTERIZATION OF PURIFIED ENZYME

a) Effect of Temperature on Enzyme Activity

Purified enzyme was characterized for the effect of temperature and the enzyme was found to be stable between 28- 37 °C. Table 7 and Figure 5 below show above said result.

Table 7: Effect of Temperature on Enzyme Activity

TEST TUBES	ENZYME (in ml)	1% STARCH (in ml)	INCUBATION TEMPERATURE	DNS (in ml)	BOIL FOR 15 MINUTES AT 100°C	O.D AT 540 nm	Enzyme Activity (U/ml/min)
BLANK	0	0	37 °C	1		0.0	00
22 °C	0.5	0.5	22 °C	1		0.09	0.0034
28 °C	0.5	0.5	28 °C	1		0.18	0.0072
37 °C	0.5	0.5	37 °C	1		0.23	0.0091
50 °C	0.5	0.5	50 °C	1		0.05	0.0019

ISSN: 0975-8585

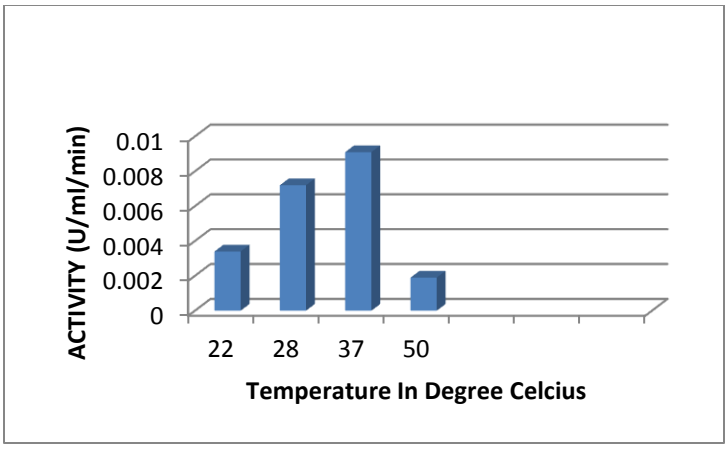

Figure 5: Effect of Temperature on Enzyme Activity

b) Effect of pH on Enzyme Activity

Purified enzyme was characterized for the effect of pH and the enzyme was found to be stable between a wide range of pH between 5 – 11 giving an indication of its extremophilic nature. Table 8 and Figure 6 below show the above said result.

Table 8: Effect of pH on Enzyme Activity

TEST TUBES	ENZYME (in ml)	1% STARCH (in ml)	INCUBATE AT 37 °C FOR 15 MINUTES	DNS (in ml)	BOIL FOR 15 MINUTES AT 100°C	O.D AT 540 nm	Enzyme Activity (U/ml/min)
BLANK	0	0		1		0.0	0.0
pH 5	0.5	0.5		1		0.21	0.0084
pH 7	0.5	0.5		1		0.23	0.0091
pH 9	0.5	0.5		1		0.21	0.0084
pH 11	0.5	0.5		1		0.16	0.0062

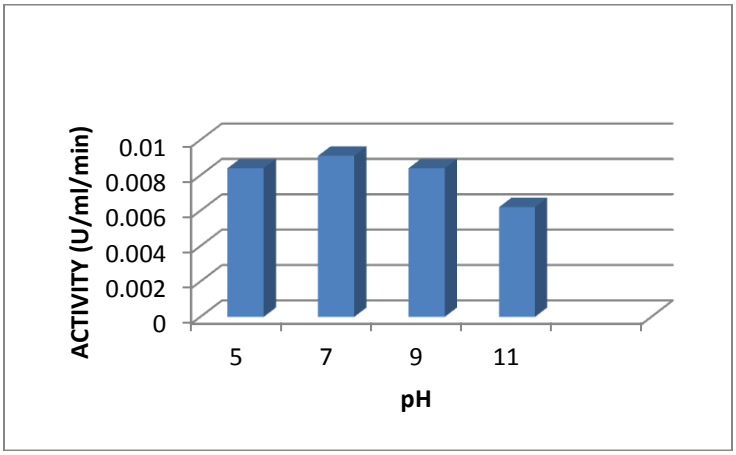

Figure 6: Effect of pH on Enzyme Activity

ISSN: 0975-8585

DISCUSSION

Microorganisms were isolated from cow dung by serial dilution agar plating method as done previously by [1].

The isolates were screened for cellulase production on minimal agar medium (pH 7) supplemented with 1% CMC, as done earlier by [1; 11, 12]. Later plates were flooded with Congo red solution and destained with 1M NaCl. Isolate showing largest cellulose hydrolysis was picked for further studies similar screening procedures have been used earlier by [13,14]. Production of cellulase was done by submerged fermentation as done previously used by [14-16]. Partial purification was subjected to fractional ammonium sulphate precipitation (upto 70% saturation). Ammonium sulphate crystals were added to the supernatant to bring the saturation in ice bath and then the suspension was dialyzed against 100mM Tris buffer of pH 7, as done earlier by [17, 18].

Enzyme activity in purified enzyme was found to be 0.008 U/ml/min which was comparable to the cellulases purified earlier. Enzyme was found to be stable in a wide pH range of 5-11, thus giving an indication of its extremophilic nature and its wide application in various industries.

CONCLUSION

Based on the above study it can be concluded that Cow dung can be a good source for the production of industrially important enzyme cellulase which play a very important role in industries like textile, paper, pharmaceutical etc. Growth of culture was found to be good at 37ºC and pH 7. The activity of partial purified cellulase here is comparable to the cellulase purified earlier by various researchers. Purified enzyme was stable in a pH range of 5-11 thus giving an indication of its extremophilic nature.

Further works includes purification of the enzyme by the help of sophisticated techniques such as ion exchange chromatography, affinity chromatography and determination of molecular weight by SDS-PAGE, characterization of the purified enzyme for the effect of activators, inhibitors and substrate concentration.

ACKNOWLEDGEMENT

We are thankful to Mr. Manoj Verma, Director, MRDLS for providing us the facilities to perform the research work. We are also thankful to all staff members of MRDLS, Lucknow for cooperation throughout the research work.

REFERENCES

[1] Ibrahim ASS and AE Diwany. J Basic and Appl Sci 2007; 1 (4): 473-478.
[2] Cavaco-Paulo A. Carbohydrate Polym 1998; 37: 273-277.

[3] Himmel ME, Ruth MF and Wyman CE. Current Opinion Biotechnol 1999; 10: 358-364.
[4] Ariffin H, Abdullah N, Kalsom MSU, Shirai Y and Hassan MA. Inter J Eng and Technol 2006; 3(1): 47-53.
[5] Gautam SP, Bundela PS, Pandey AK, Jamaluddin MK, Awasthi and Sarsaiya S. Int J Academic Res 2010; 2(6): Part II.
[6] Baharuddin AS, Razak MNA, Hock LS, Ahmad MN, Aziz SA, Rahman NAA, Shah UKM, Hassan MA, Sakai K and Shirai Y. American Journal of Applied Sciences 2010; 7(1): 56-62.
[7] Otajevwo FD and Aluyi HSA. Nigerian Journal of Microbiology 2010; 24(1): 2168 – 2182.
[8] Aneja KR. Experiment in microbiology, plant pathology and biotechnology, Fourth Edition. New Age International (p).Ltd. Publishers, New Delhi, 2003.
[9] Lowry OH, Rosebrough AL and Farr RJR. J Biol Chem 1951; 193-256.
[10] Miller GL. Anal. Chem 1959; 31:426-428.
[11] Alam MZ, Manchur MA and Anwar MN. Pakistan J Biol Sci 2004; 7(10): 1647-1653.
[12] Kotchni SO, Gachomo EW, Omafuvbe BO and Shonukam OO. Int J Agri Biol 2006; 8(2): 286-292.
[13] Corderio CAM, Martins MLL, Luciano AB and Silv RFD. Brazilian Arch Biol Technol 2002; 45(4): 413-418.
[14] YC Lo, Saratalea GD, Chend WM, Basic MD and Changa JS. Enz Microbial Technol 2009; 44: 417-425.
[15] XH Li, Bhaskar R, Yang HJ, Wang D and Miao YG. Curr Microbiol 2009; 59: 393- 399.
[16] Govender L, Naidoo L and Setati ME. African J. Biotechnol 2009; 8(20): 5458-5466.
[17] Maki LM, Broere M, Leung KT and Qin W. J Biochem Mol Biol 2011; 2(2): 146-154.
[18] Odeniyi OA, Onilude AA and Ayodele MA. African J Microbiol Res 2009; 3(8): 407-417.